Journal of Physiology (1993), **462**, *pp.* 563–578
With 6 figures
Printed in Great Britain

GUANINE NUCLEOTIDE BINDING PROTEINS MEDIATE D_2 DOPAMINE RECEPTOR ACTIVATION OF A POTASSIUM CHANNEL IN RAT LACTOTROPHS

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(*Received 1 November 1991*)

SUMMARY

1. The involvement of guanine nucleotide binding proteins in the coupling of D_2 dopamine (DA) receptors to single potassium channels was examined in rat pituitary lactotrophs.

2. Lactotrophs were unambiguously identified by the reverse haemolytic plaque assay (RHPA) and membrane potentials, whole-cell and single channel currents recorded using patch electrode methods.

3. DA or the D_2 selective agonist, quinpirole, induced the opening of single K^+ channels in cell-attached patches underlying robust hyperpolarizations of membrane potential in single cells.

4. Both whole-cell and single channel responses were independent of Ca^{2+} or cAMP concentrations.

5. Pertussis toxin (PTX) pretreatment (50–250 ng/ml, 6–12 h) blocked the action of DA on lactotroph membrane potential and uncoupled D_2 receptors from single K^+ channels in cell-attached patches.

6. Internal dialysis with GDPβS (guanosine 5′-*O*-(2-thiodiphosphate) greatly reduced whole-cell responses to DA in a dose-dependent manner.

7. Internal dialysis of lactotrophs with GTPγS (guanosine 5′-*O*-(3-thiotriphosphate) potentiated DA responses in a dose-dependent manner while rendering the responses irreversible at higher doses.

8. DA (100 nM) or quinpirole (10 μM) activated K^+ channels in excised outside-out membrane patches that were identical to those identified in cell-attached patches in terms of conductance and gating kinetics.

9. It is proposed that D_2 receptors are coupled to non-voltage-dependent K^+ channels by G proteins of the G_i/G_o class and that this coupling is via a direct, membrane delimited pathway.

INTRODUCTION

In the central nervous system many of the physiological actions of dopamine (DA) are mediated by its interaction with inhibitory, dopamine D_2 receptors. In the anterior pituitary, DA is well established as the primary regulator of prolactin (PRL) secretion by exerting a tonic inhibitory control (for review see Ben-Jonathan, 1985).

DA inhibition of PRL secretion has been demonstrated to be mediated via D_2 receptors present on the prolactin-secreting cells of the pituitary (i.e. lactotrophs). The coupling of these receptors to a pertussis toxin (PTX)-sensitive guanine nucleotide binding protein (G protein) appears to play a necessary role in the dopaminergic inhibition of secretion (Caron *et al.* 1978; Cronin, Meyers, MacLeod & Hewett, 1983; Enjalbert & Bockaert, 1983; Enjalbert, Mussett, Chenard, Priam, Kordon & Heisler, 1988). The mechanisms by which DA inhibits PRL secretion at a cellular level are still a matter of dispute. In the pituitary D_2 receptors have been demonstrated to negatively couple to several effector systems including adenylyl cyclase and phosphoinositide metabolism, and stimulate potassium (K^+) channels (for review see Vallar & Meldolesi, 1989). Like neurons, pituitary cells display spontaneous electrical activity including Ca^{2+}-dependent action potentials which are involved in stimulus–secretion coupling. Interestingly, several groups have reported that stimulation of D_2 receptors present in lactotrophs results in an inhibition of Ca^{2+}-dependent action potentials due to a hyperpolarization of membrane potential thought to be mediated by an increase in K^+ conductance (Israel, Jaquet & Vincent, 1985; Ingram, Bicknell & Mason, 1986; Einhorn, Gregerson & Oxford, 1991).

Previous studies have established that D_2 receptors in the central nervous system and pituitary are both physically and functionally coupled to guanine nucleotide binding proteins (G proteins) (DeLean, Kilpatrick & Caron, 1982; Kilpatrick & Caron, 1983; George, Watanabe, Di Paola, Farlardeau, Labrie & Seeman, 1985; Senogles, Spiegel, Padrell, Iyengar & Caron, 1990). The cDNA and gene for the rat brain D_2 receptor have been cloned and found to encode two proteins (isoforms) which possess many of the structural characteristics common to members of the G protein-coupled receptor family (Bunzow *et al.* 1988; Giros, Sokoloff, Martes, Riou, Emorine & Schwartz, 1989; Grandy *et al.* 1989; Monsma, McVittie, Gerfen, Mahan & Sibley, 1989; Strader, Sigal & Dixon, 1989). While G proteins were initially thought to be primarily involved in the regulation of second messenger systems, it has become evident that G proteins are intimately involved in the regulation of ion channels (Breitwieser & Szabo, 1985; Pfaffinger, Martin, Hunter, Nathanson & Hille, 1985; Holz, Rane & Dunlap, 1986; Lewis, Weight & Luini, 1986; Yatani, Codina, Sekura, Birnbaumer & Brown, 1987; Ewald, Pang, Sternweis & Miller, 1989). In fact, evidence suggests that G proteins may directly couple to K^+ channels as in the case of the muscarinic-activated K^+ channel in heart (e.g. Logothetis, Kurachi, Galper, Neer & Clapham, 1987). Furthermore, DA regulation of K^+ conductance in rat substantia nigra (Lacey, Mecuri & North, 1988) and *Aplysia* neurons (Sasaki & Sato, 1987) is thought to be linked to G proteins. Using the reverse haemolytic plaque assay to identify primary rat lactotrophs in combination with whole-cell and single channel patch clamp techniques, we have investigated the role of G proteins in the coupling of D_2 receptors to effector K^+ channels. The results of the present study suggest that the anterior pituitary D_2 receptors are coupled to K^+ channels through the activation of G proteins, and that this coupling may be 'direct', that is, without acting through a soluble second messenger. These findings raise the possibility that DA inhibits PRL secretion through a DA-induced hyperpolarization of resting membrane potential mediated by the 'direct' G protein coupling of D_2 receptors to the activation of a specific class of K^+ channels.

METHODS

Anterior pituitary dissociation

Adult female rats in the pro-oestrus phase of the oestrous cycle were decapitated under methoxyfluorane anaesthesia and the anterior pituitary glands removed on ice by dissection. Cells were dispersed enzymatically using a non-trypsin dissociation protocol modified from that of Weiner, Bethea, Jaquet, Ramsdell & Gospodarowicz (1983). In brief, the glands were removed and minced in sterile Hanks' balanced salt solution, calcium and magnesium free (Hanks' CMF). After washing the fragments were incubated in a shaking water bath for 1 h at 37 °C in Hanks' CMF containing trypsin inhibitor (0·1 mg %), collagenase (Worthington Biochemicals, 0·3 mg %) and DNase I (1 mg %). Fragments were then mechanically dispersed by trituration with a siliconized Pasteur pipette, washed with Dulbecco's modified Eagle's medium containing 0·1 % bovine serum albumin (DMEM-BSA), filtered through a nylon mesh (20 μm) and harvested via centrifugation.

Reverse haemolytic plaque assay

The reverse haemolytic plaque assay (RHPA) was performed as a means to unambiguously identify functionally secreting lactotrophs in a mixed cell culture. A detailed description of the procedure has been reported elsewhere (Smith, Luque & Neill, 1986) as well as modifications of our own (Einhorn *et al.* 1991). Immediately following the dissociation, pituitary cells suspended in DMEM-BSA (4×10^5/ml) were mixed with an equal volume of a 9 % suspension of ovine erythrocytes previously coupled to *Staphylococcus* protein A with aged chromium chloride hexahydrate (0·9 mg/ml). Aliquots of this mixture were infused into modified Cunningham chambers which were constructed by affixing a polylysine-coated glass coverslip to a microscope slide by means of two parallel pieces of double-sided tape. The chambers were then rinsed with DMEM-BSA and the assay initiated by infusion of DMEM-BSA containing rabbit antiserum to rat PRL (PRL-1-5 generated in our laboratory, 1:120 dilution) and placed in the incubator for 1 h. The plaques were developed by infusion of guinea-pig complement (1:50–1:100 dilution, 30 min). The complement reaction was terminated by repeated rinses with DMEM-BSA and the chambers were dismantled. The coverslips with cells attached were maintained in culture in DMEM containing 10 % equine serum and gentamycin (40 μg/ml) for electrophysiological experiments 1–3 days following the assay.

Electrophysiological procedures

Whole-cell and single channel recordings were made using gigaohm-seal patch clamp techniques as described by Hamill, Marty, Neher, Sakmann & Sigworth (1981). Patch electrodes with 2–6 MΩ tip resistances were constructed from N51A capillary glass (Drummond Scientific, Broomall, PA, USA) and coated with Sylgard (Dow Corning). Recordings were made using an Axopatch 1A patch clamp (Axon Instruments) and macroscopic currents were low-pass filtered at 1–2 kHz with a Bessel filter. Whole-cell macroscopic currents were sampled at 12-bit resolution by an A–D converter (Scientific Solutions LabMaster), the data were stored on an 80286-based microcomputer (Dell Computer Systems), and analysed with custom programs written in C (C-Lab, INDEC). Single channel currents as well as whole-cell voltage responses were recorded on videotape using a digital audio processor interface at full bandwidth (44 kHz). Stored videotape recordings were redigitized by a computer for subsequent analysis of single channel current data using Axotape (version 1.2) and pClamp (version 5.5) software (Axon Instruments).

The basic extracellular solution for whole-cell and cell-attached single channel recording consisted of (mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 Hepes buffer adjusted to pH 7·4 and osmolarity 295–300 mosmol/l. In some experiments K^+ currents were isolated with solutions containing TTX (1 μM), EGTA (1 mM), and $CdCl_2$ (100 μM) while omitting $CaCl_2$. For outside-out single channel recording the extracellular solution was modified to contain 20 mM KCl and 120 mM NaCl in order to increase the inward driving force on K^+.

The composition of the internal solutions varied according to the specific nature of the experiment. The basic pipette solution for whole-cell recordings consisted of (mM): 130 potassium aspartate, 20 KCl, 10 glucose, and 10 Hepes buffer adjusted to pH 7·4 and osmolarity 300–305 mosmol/l. In initial whole-cell experiments solutions also contained 2 mM cAMP, 2 mM MgATP, and 100 μM GTP. In some cases guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) were substituted for GTP where indicated. For cell-attached single channel experiments the basic pipette solution contained 150 mM KCl, 3·1 mM $MgCl_2$ and 15·5 mM Hepes. The pipette solution for outside-out patch recording contained (mM): 130 potassium

aspartate, 20 KCl, 10 EGTA, 10 Hepes, 10 glucose, 2 MgATP, 2 cAMP and 0·4 GTP. Drugs were made freshly each day just prior to experiments. DA was prepared as a stock solution and diluted with ascorbic acid in 100-fold excess to prevent oxidation. Quinpirole (Research Biochemicals Inc.) was diluted from a stock solution in dimethyl sulphoxide (DMSO) to a final DMSO concentration of $\leqslant$0·5 %. All experiments were performed at room temperature and the external bath solution was continuously perfused into the recording chamber by gravity flow. Application of drugs was accomplished through a U-tube device which could be positioned next to a cell to rapidly (time constants $<$20–30 ms) apply and withdraw a solution (Oxford & Wagoner, 1989).

RESULTS

Characteristics of whole-cell and single channel responses to DA

The hallmark effects of DA on the electrical properties of RHPA-identified lactotrophs are illustrated in Fig. 1 and have been described in detail previously (Gregerson, Einhorn & Oxford, 1989; Einhorn *et al.* 1991). The effect of DA on the membrane potential in identified lactotrophs was examined using the whole-cell current clamp technique. In a lactotroph which was not spontaneously active, a brief application of DA (100 nM) evoked a large (15–20 mV), rapidly developing hyperpolarization of the membrane potential which recovered to baseline within 2 min following removal of the agonist (Fig. 1*A*). Attenuated membrane potential responses to hyperpolarizing current pulses (−5 pA indicated by the asterisks) during the DA-induced hyperpolarization indicate a large ($\approx$ 50 %) reduction in cell input resistance. This electrophysiological effect does not appear to involve a D_2 receptor-mediated inhibition of cAMP, as robust hyperpolarizations (e.g. Fig. 1*A*) could be elicited by DA when the pipette solution contained 2 mM cAMP, effectively 'clamping' the intracellular cAMP concentration.

The DA-activated whole-cell current was examined under voltage clamp. At a membrane potential of −30 mV, DA elicited an outward current of 10–20 pA (for example Fig. 1*B*). In addition the current–voltage relationship obtained by ramp voltage commands (−120 to +20 mV) revealed that the DA-induced current reversed at −72 mV which is coincident with the calculated equilibrium potential for K^+ (Fig. 1*C*). When applied in the external bathing solution, the K^+ channel blocker quinine (100 μM) completely eliminated the inward current and inhibited the outward current (Fig. 1*C*). Furthermore, the DA-activated K^+ current was not calcium (Ca^{2+}) dependent as the current could be elicited when both the extracellular and intracellular Ca^{2+} was buffered with EGTA to negligible levels (Einhorn *et al.* 1991).

Single DA-activated K^+ channels were identified using the cell-attached recording configuration. When DA (100 nM) or the selective D_2 agonist, quinpirole (10 μM), was included in the pipette solution we observed single channel currents which were rarely seen in the absence of agonist (Figs 1*D* and 5). Analysis of current–voltage relationships for D_2 receptor-activated K^+ channels yielded single channel conductances of 40–50 pS. Open dwell-time histograms of the DA-activated channel openings were best fitted by single exponential functions, indicating that the channel exhibited only one brief open state with an average of 0·85 ms that was independent of voltage. Details of this analysis have been published previously (Einhorn *et al.* 1991).

Interference with inhibitory G proteins reduces whole-cell responses to DA

To investigate the possible role of G proteins in the whole-cell response to DA, we first examined the action of PTX on the DA-induced hyperpolarization. We chose to

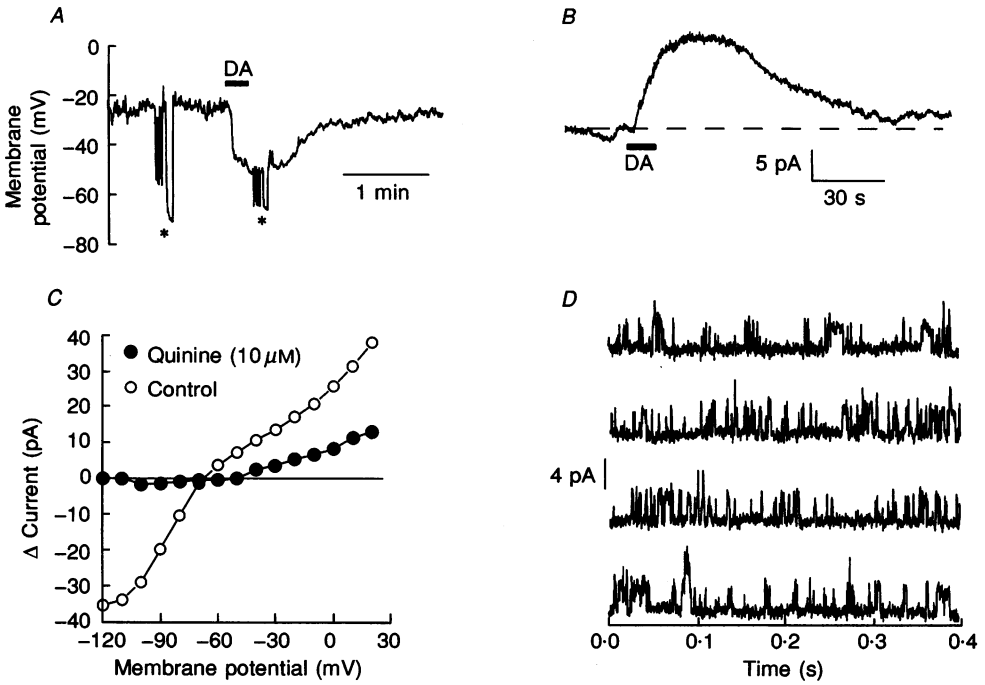

Fig. 1. Typical whole-cell and single channel responses to DA of primary rat lactotrophs. *A*, membrane potential response to DA in whole-cell current clamp. Brief (bar) application of DA (100 nM) elicited a rapidly developing hyperpolarization. Attenuated voltage responses to injection of 5 pA hyperpolarizing current pulses (*) before and at the peak response indicate a decreased membrane resistance. *B*, current response to DA in whole-cell voltage clamp. Membrane potential was held at −40 mV and a brief application (bar) of DA (100 nM) elicited a 15 pA outward current. *C*, current–voltage relationship of the DA-induced current. Voltage was commanded by ramps from −120 to +20 mV and the current responses to three ramps were averaged. Average responses during a control period were subtracted from those obtained at the peak DA (100 nM) response to yield the DA-sensitive current. The DA-activated current reversed near the equilibrium potential for K^+ and was blocked by an external application of quinine (100 μM). *D*, cell-attached single channel recordings of D_2 agonist-activated K^+ channels. Representative records of channel activity in a patch exposed to quinpirole, a selective D_2 agonist, in the patch electrode. Pipette potential was held at +60 mV. The electrode solution contained 150 mM K^+. Upward deflections represent inward (pipette to cell) current.

examine membrane voltage responses rather than the current responses owing to the natural amplification of small current changes by the high input impedance of the cells (≈ 8 GΩ; see Einhorn *et al.* 1991). PTX catalyses the ADP ribosylation of specific classes of G proteins, G_i and G_o, thereby preventing their activation by agonist (Bokoch, Kadat, Northup, Hewlett & Gilman, 1983; Murayama & Ui, 1983; Kadata, Northup, Bokoch, Ui & Gilman, 1984). Preincubation of cells with PTX (50–250 ng/ml, overnight) abolished the ability of DA to elicit a hyperpolarization of membrane potential (e.g. Fig. 2*B*). Whereas 85 % of control cells responded to DA with an average −20 mV hyperpolarization, only one out of eighteen PTX-treated cells we examined responded with a change in membrane potential, and this response was markedly reduced (Fig. 3).

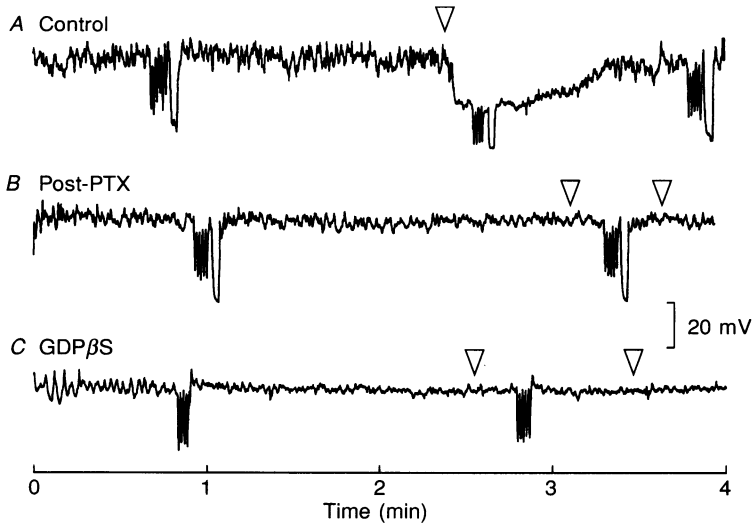

Fig. 2. Effects of pertussis toxin and GDPβS on voltage responses of lactotrophs to DA. Recordings from three cells represent typical DA responses in a control cell (*A*) and the absence of DA responses in cells pretreated with 250 mg/ml PTX overnight (*B*) or dialysed with 500 μM GDPβS (*C*). Brief applications of DA (100 nM, 2 s) are indicated by inverted arrowheads. Occasional transient downward interruptions of the traces are responses to a series of −5 pA current injections of different durations to assess membrane resistance.

The involvement of G proteins was further revealed by experiments in which single cells were internally dialysed with GDPγS, the non-hydrolysable analogue of GDP. GDPβS is thought to block the actions of G proteins by competitively inhibiting the binding of GTP necessary for G protein activation. Lactotrophs internally dialysed with GDPβS (n=17, 100–500 μM), by inclusion in the pipette solution during whole-cell recording, were substantially less responsive to DA when compared to controls (e.g. Fig. 2*C*). This reduction was dose dependent and reflected as both a reduction in the number of cells responding and the amplitude of the hyperpolarization (Fig. 3). Although control cells were normally dialysed with GTP (100 μM), we observed that exclusion of GTP from the internal solution did not result in any notable instability of membrane potential nor were DA responses altered in any obvious way.

Effects of G protein activation on the whole-cell response to DA

The non-hydrolysable analogue of GTP, GTPγS, was also employed to assess the role of G proteins in mediating these events. GTPγS is thought to activate G proteins by competitively displacing GTP from its binding site on the α-subunit and thus preventing the nucleotide hydrolysis that normally terminates G protein activation. In the absence of DA, intracellular dialysis with GTPγS (n=8, 50–200 μM) resulted in a slowly developing membrane hyperpolarization which was maximal by 7–10 min after establishment of the whole-cell recording configuration. (e.g. Fig. 4*B*). The GTPγS-induced potential change was comparable to that produced by DA in both amplitude (20 mV) and associated increase in membrane conductance. Furthermore, application of DA to a GTPγS-hyperpolarized cell resulted in little or no additional change in membrane potential (Fig. 4*B*). In

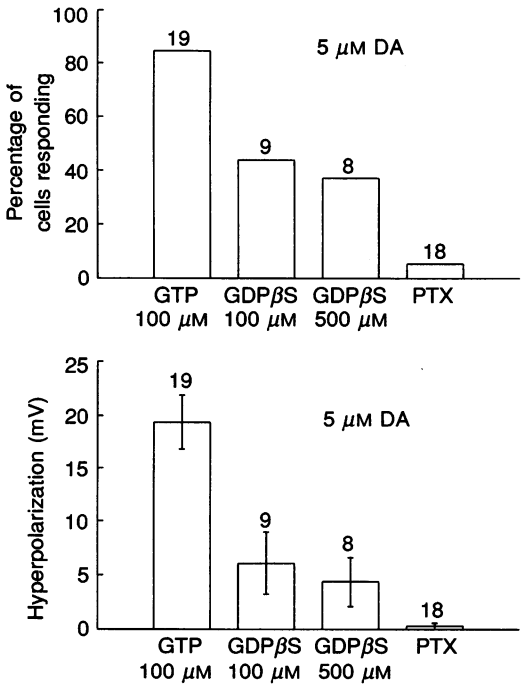

Fig. 3. Sensitivity of the DA-induced hyperpolarization to the block of G protein activation. Summary of whole-cell current clamp experiments represents the percentage of lactotrophs responding to DA (5 μM) and the maximum hyperpolarization of those responses (means ± S.E.M.). Control and PTX-treated (250 ng/ml, overnight) cells were exposed to 100 μM GTP in the internal solution. Other cells had 100 or 500 μM GDPβS added internally.

contrast, during control experiments in which cells were dialysed with the hydrolysable GTP, a spontaneous hyperpolarization in membrane potential was never observed and the potential remained stable for many tens of minutes.

In some experiments, cells dialysed with GTPγS were challenged with DA shortly after establishment of the whole-cell recording mode, before a GTPγS effect fully developed (Fig. 4*C* and *D*). When cells (n=4) were dialysed with GTPγS (50 μM) repeated applications of DA resulted in hyperpolarizations which only partially recovered and appeared to decrease in size as the response accumulated (e.g. Fig. 4*C*). When cells (n=6) were dialysed with a higher concentration of GTPγS (150–200 μM), a single application of DA resulted in a rapid and irreversible hyperpolarization (e.g. Fig. 4*D*). While multiple reversible agonist-induced hyperpolarizations were routinely observed in control GTP-dialysed cells, a single 10 s application of DA to these GTPγS-dialysed cells was usually followed by a hyperpolarization that did not recover during the 20–30 min of the recording session.

Effects of PTX on single D_2 receptor-activated K^+ channels

The role of G proteins in the coupling of D_2 receptor to K^+ channels was also investigated at the single channel level using cell-attached membrane patches. Cells were pretreated with PTX as described for whole-cell experiments and challenged with DA (100 nM) by inclusion in the pipette solution. DA-activated K^+ channels, as

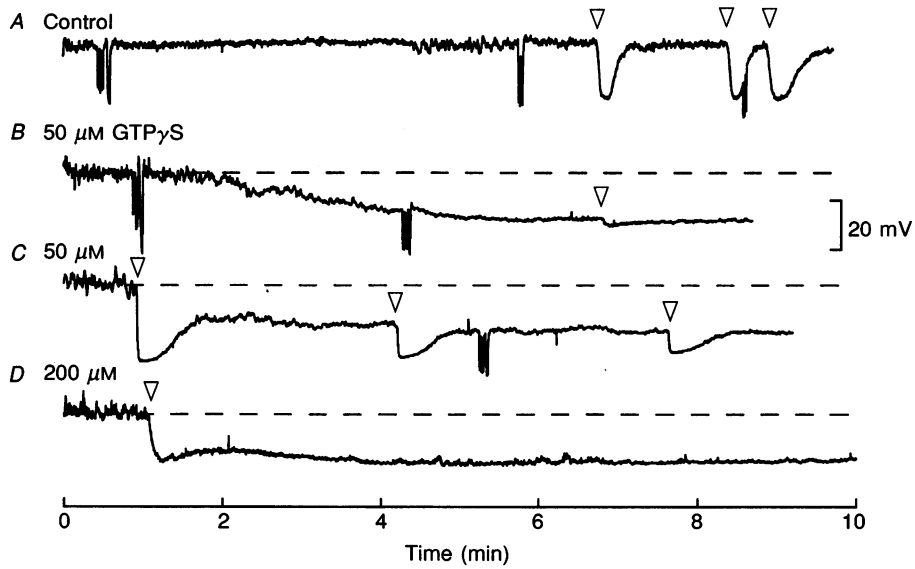

Fig. 4. Effects of internal dialysis with GTPγS on voltage responses of lactotrophs to DA. Brief DA (100 nM) applications are indicated by arrowheads in each trace. Occasional injections of −5 pA current pulses were used to assess membrane resistance. *A*, three consecutive DA responses were elicited in a control cell dialysed with GTP (100 μM) alone. *B*, dialysis with 50 μM GTPγS resulted in a slow spontaneous hyperpolarization to a level similar to that achieved in the control cell during DA application. Subsequent application of DA to this cell produced only a small additional change (arrowhead). *C*, repeated applications of DA to a cell in the early stages of dialysis with 50 μM GTPγS resulted in rapid hyperpolarizations which partially recovered and appeared to decrease in size as the response accumulated. *D*, in a cell dialysed with 200 μM GTPγS early application of DA elicited a rapid hyperpolarization which did not recover within 10 min following removal of DA.

previously described, were observed in 80 and 85 % of control patches exposed to DA ($n=19$) or the selective D_2 agonist quinpirole (10 μM, $n=15$), respectively. In contrast when receptor–G protein interactions were uncoupled by pretreatment with PTX, DA-induced channel activity was observed in only 15 % ($n=8$) of membrane patches. Similar effects were observed when K^+ channel activation was blocked at the level of the receptor by exposure of cell-attached patches to quinpirole in the presence of the selective D_2 antagonist sulpiride (25 μM). Under these conditions only 20 % ($n=9$) of the patches exhibited any channel activity.

DA-activated K^+ channels in outside-out membrane patches

The outside-out patch configuration was employed in order to test the possibility that D_2 receptors might directly couple to K^+ channels via G proteins without acting through soluble second messengers. Given the substitution of an 'infinite' volume of the patch electrode solution for the normal cytosol, activation of soluble second messenger mechanisms is extremely improbable. Single channel recordings were made in outside-out patches ($n=6$) in which GTP (400 μM) was included in the pipette solution. In the absence of D_2 receptor stimulation, current recordings yielded only an occasional opening of a single channel (Fig. 5*A*). During a 10–15 s application of either DA (100 nM) or quinpirole (10 μM) to the extracellular face of the

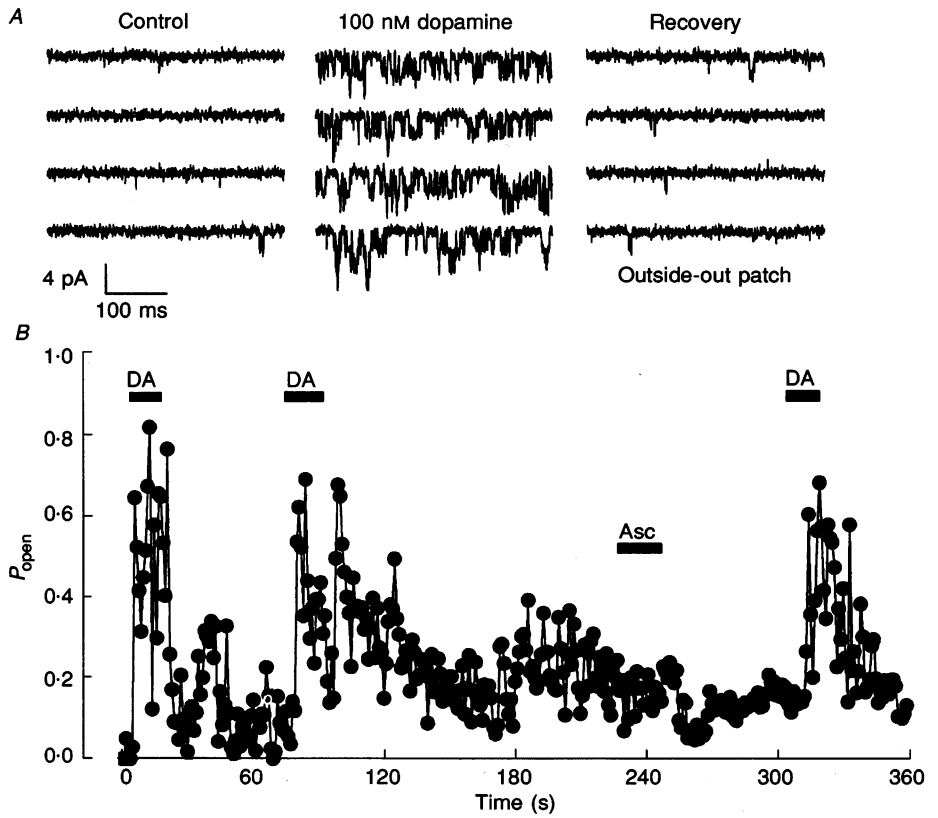

Fig. 5. DA-activated K^+ channels in outside-out membrane patches. Outside-out single channel recordings of DA-stimulated K^+ channels were made with pipette solutions containing 400 μM GTP in addition to the basic intracellular solution (see experimental procedures). Membrane potential was held at −120 mV. *A*, representative records of basal channel activity prior to DA (control) are compared to records of channel acivity during 15 s application of 100 nM DA. Channel activity had recovered to baseline 20 s after termination of the DA application. Current recordings were low-pass filtered at 2 kHz and data sampled at 5 kHz. *B*, sequential responses of channels in an outside-out patch to repeated applications of 100 nM DA (indicated by bars). Running open probabilities (P_{open}) were calculated for sequential 1 s epochs of a continuous recording from the percentage channel open time divided by the number of channels estimated in the patch (three) from observations of simultaneous openings. Note the absence of a response to the application of the ascorbate vehicle (Asc).

patch, frequent channel openings were observed. In general, a patch contained more than one channel as simultaneous openings were occasionally observed. Following the withdrawal of agonist, channel openings became less infrequent and baseline activity was recovered within 30–60 s. Figure 5*B* illustrates the responses (estimated open probability) of a single patch containing three channels to several DA challenges. Channel open probability increased with only a brief delay (1–2 s) of the onset of agonist application, but diminished more slowly following abrupt ($\leqslant$ 100 ms) removal of agonist by the U-tube system. The absence of a response to the ascorbate-containing control solution indicates these opening events are receptor mediated.

Amplitude histograms of DA-activated channel openings were constructed to determine the mean current amplitude at different voltages. Analysis of the resulting current–voltage relationships yielded a single channel conductance of 42 pS, a value similar to that reported previously for DA-activated channels in cell-attached patches (Einhorn *et al.* 1991). Open time histograms of DA-activated channel openings in outside-out patches were also similar to those observed in cell-attached patches (data not shown). Opening distributions were best fitted to a single exponential function with a time constant of 0·83 ms.

DISCUSSION

D_2 receptors on PRL-secreting cells mediate dopaminergic inhibition of prolactin secretion (Caron *et al.* 1978; Enjalbert & Bockaert, 1983). Furthermore, the coupling of D_2 receptors to a PTX-sensitive G protein also appears to play a necessary role in this regulation (Cronin *et al.* 1983). The present studies were conducted in order to investigate the role of G proteins in the coupling of D_2 receptors to effector K^+ channels in primary rat lactotrophs. Our focus on K^+ channels reflects the evidence from the literature and our own work that a K^+ conductance plays the central role in the inhibition of prolactin secretion by DA (Israel, Jaquet & Vincent, 1985; Ingram *et al.* 1986; Castaletti, Memo, Missale, Spano & Valerio, 1989; Lledo, Legendre, Israel & Vincent, 1990; Einhorn *et al.* 1991) Our results confirm and extend these findings to the single channel level. Regulation of K^+ channels by D_2 receptors may be a more common phenomenon as it has also been reported for melanotrophs from the intermediate lobe (Stack & Suprenant, 1991), substantia nigra and striatal neurons from rat (Lacey, Mercuri & North, 1987; Freedman & Weight, 1988), as well as abdominal gangion neurons of *Aplysia* (Sasaki & Sato, 1987).

PTX treatment results in the selective ADP ribosylation of G proteins of the G_i/G_o classes, and thus blocks the ability of these proteins to couple to receptors and dissociate into their active subunits. PTX treatment of rat lactotrophs blocked both the DA-induced hyperpolarization of membrane potential as well as DA activation of single K^+ channels.

Additional support for the involvement of G proteins was revealed by examining the effects of GDPβS and GTPγS on DA responsiveness. GDPβS-dialysed cells were significantly inhibited in their ability to respond to DA with changes in membrane potential indicating a functional uncoupling of D_2 receptors from K^+ channels. Alternatively the interaction between the guanine nucleotide and the G protein species associated with the D_2 receptor is known to convert the receptor to a lower agonist affinity state. This could masquarade as an uncoupling at moderate agonist concentrations. Our observations of diminished responses to 5 μM DA are well above K_D values reported for D_2 receptors in either the presence or absence of guanine nucleotides. The DA-induced hyperpolarization was also mimicked by intracellular GTPγS in the absence of DA. The observation that DA responses and GTPγS responses in a single cell were not additive (e.g. Fig. 4*B*) suggests that GTPγS bypasses the D_2 receptor to activate the same G proteins in the receptor–channel signal transduction pathway. The slowly developing nature of the GTPγS-induced hyperpolarization in the absence of agonist is thought to reflect the rate-limiting basal release of GDP from the G protein. However, as GDP–GTPγS exchange at the guanine nucleotide binding site is agonist driven, when DA was applied to GTPγS-

dialysed cells, changes in membrane potential became extremely rapid and irreversible. Furthermore, the effect of GTPγS on the DA response was concentration dependent. When cells were dialysed with a lower concentration of GTPγS the repeated DA-induced potential changes only partially recovered leading to a cumulative hyperpolarization with each application of agonist.

The receptor–G protein–K^+ channel coupling mechanism documented here is consistent with some other reports for DA regulation of K^+ conductances described for endocrine cells as well as neurons. Using voltage-sensitive dyes to measure changes in membrane potential, Malgaroli, Vallar, Elahi, Pozzan, Spada & Meldolesi (1987) demonstrated that PTX pretreatment blocked the D_2 receptor-mediated hyperpolarization of membrane potential in primary cultures enriched in lactotrophs. Sasaki & Sato (1987) reported that a DA-activated K^+ conductance was also blocked by PTX and mimicked by GTPγS in *Aplysia* neurons. Furthermore, while Lacey *et al.* (1988) reported that hyperpolarizations induced by DA in substantia nigra neurons were not affected by prior intracerebroventricular injection of animals with PTX, microinjection of GTPγS into the neurons blocked recovery of membrane potential following application of DA.

Interestingly, we observed that inclusion of GTP in the pipette solution was not an absolute requirement for DA-induced K^+ currents or hyperpolarizations. Such a finding does not, however, preclude the hypothesis that G proteins mediate the electrical responses to DA. Similar findings have been reported for the G protein-coupled, inwardly rectifying K^+ channel activated by acetylcholine in rabbit and bullfrog cardiac tissue (Soejima & Noma, 1984; Breitwieser & Szabo, 1988). In fact, it has been suggested that the levels of intracellular GTP during whole-cell dialysis are probably in the order of 100-fold greater than that necessary for GTP-mediated processes (Breitwieser & Szabo, 1988).

While many of our findings parallel those reported for the muscarinic-activated K^+ channels in cardiac tissues, one observation reflects perhaps an important difference in the signal transduction kinetics in the two cases. From the experiments of Breitwieser & Szabo (1988) it is apparent that both the activation (agonist applied) and deactivation (upon agonist removal) of the current induced by acetylcholine are rapid responses.

The deactivation response rate is thought to reflect primarily the rate of GTP hydrolysis by the salient G protein species. In contrast, the DA response in lactotrophs persists well beyond removal of the agonist by the U-tube system as illustrated at the single channel level in an outside-out patch (Fig. 6) upon DA (100 nM) application and withdrawal. This observation suggests differences in either the relative dissociation rates of DA and ACh from their respective receptors, or differences in the kinetics of the G protein–receptor–effector cycles in the two cases. Further studies are planned to address this issue.

While previous investigations have established that G proteins are intimately involved in the regulation of ion channels, it is becoming evident that the mechanisms by which they exert their effects are complex (e.g. Kim, Lewis, Graziadei, Neer, Bar-Sagi & Clapham, 1989). In some systems G proteins couple to effector ion channels through their activation of second messenger systems such as the noradrenergic regulation of Ca^{2+} channels in heart muscle and dorsal root ganglion neurons (Holz *et al.* 1986). In other cases, such as the muscarinic-stimulated K^+ channel in heart, soluble second messengers are apparently not required for

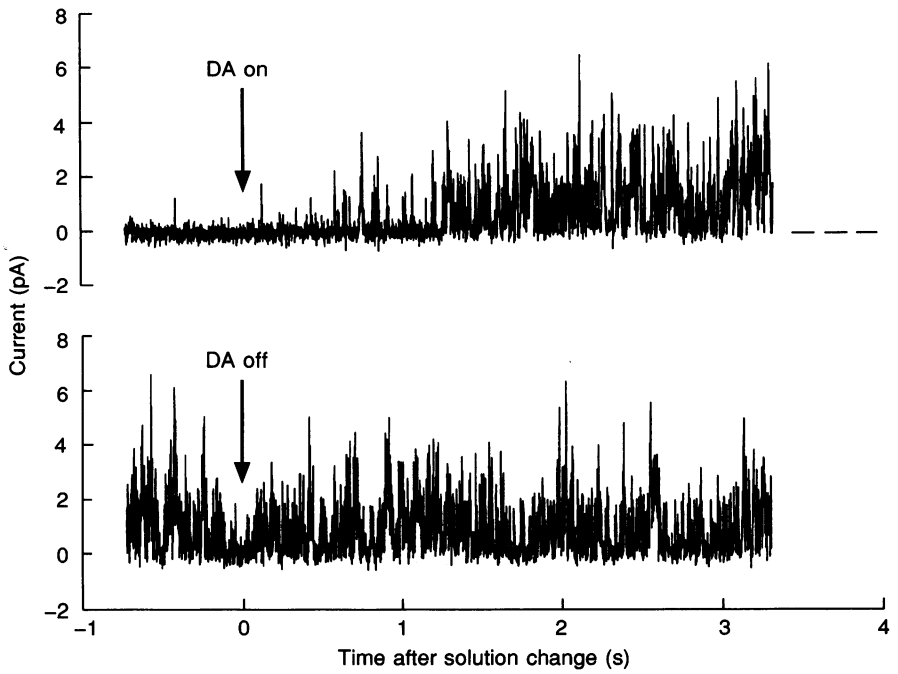

Fig. 6. Kinetics of activation and deactivation of DA K^+ channel responses in an outside-out membrane patch. Recordings of single channel activity under the same conditions as for Fig. 5. In the upper trace channels activate with a brief 1–2 s delay following DA application (arrow). The lower trace is a segment recorded later from the same patch in which the DA application is terminated (arrow), yet channel activity is sustained for several seconds.

channel activation, and thus this type of regulation has been referred to as 'direct' (Pfaffinger *et al.* 1985; Logothetis *et al.* 1987).

Several of our findings presented in this study are consistent with the hypothesis that D_2 receptors in lactotrophs are *directly* coupled to K^+ channels. D_2 receptor stimulation in the anterior pituitary, is linked to inhibition of both adenylyl cyclase activity and phosphoinositide metabolism (for review see Vallar & Meldolesi, 1989). In most of our whole-cell experiments the level of intracellular cAMP was effectively 'clamped' at 2 mM by inclusion of cAMP in the pipette solution. In other experiments Ca^{2+} was reduced to negligible levels by buffering both the internal and external solutions with EGTA. Under these conditions, DA remained capable of eliciting robust electrical responses indicating that DA responsiveness was independent of Ca^{2+} concentration as well as D_2 regulation of adenylate cyclase and corresponding cAMP levels. In addition, single D_2 agonist-activated K^+ channels were recorded in outside-out membrane patches which were essentially identical to those observed in cell-attached patches in both conductance and gating properties. We know of only one other report (Kim, 1991) of agonist-activated channel activity in an outside-out patch for any G protein-regulated channel. As soluble second messengers and other cytoplasmic factors are presumably lost and/or diluted during the formation of the outside-out patch, the coupling of D_2 receptor to K^+ channels therefore appears to involve membrane delimited processes. It is conceivable that

the D_2 receptor is itself an agonist-gated ion channel like the nicotinic acetylcholine receptor. This alternative view is unlikely, however, owing to the significant delays between agonist presentation and channel activation beyond those expected from solution exchange limits. Ultimately, the direct coupling hypothesis will need to be explored further by testing the ability of DA to stimulate channel activity in excised, inside-out membrane patches, and any channel-forming abilities of purified D_2 receptors reconstituted into bilayers should be examined as well.

While it is not yet possible to provide direct experimental evidence for a causal link between the DA-activated K^+ channels and inhibition of PRL secretion, our observations strongly support a role for a K^+ conductance in the process. The correlations between electrical and secretory responses to DA are striking. As investigators have demonstrated that stable cAMP concentrations do not prevent the inhibition of PRL secretion, both inhibitory responses appear independent of intracellular levels of cAMP (Tam & Dannies, 1981; Delbeke, Scammel, Martinez-Campos & Dannies, 1986; Ray, Gomm, Law, Sigournay & Wallis, 1986). Secondly, G proteins play a significant role in the signal transduction pathways for both responses. In fact, pretreatment with PTX, which inhibits a certain class of G proteins abolishes DA activation of K^+ channels as well as DA inhibition of PRL secretion. Furthermore both responses are mediated by D_2 dopamine receptors and are associated with decreases in intracellular Ca^{2+} (Malgaroli *et al.* 1987; Mason *et al.* 1989). An alternative mechanism that has been suggested is an inhibition of voltage-gated Ca^{2+} influx believed to support basal PRL secretion during spontaneous electrical activity. Lledo *et al.* (1990) have reported such inhibition by dopamine of two classes of Ca^{2+} currents in lactotrophs from lactating female rats maintained in culture for more than 1 week. We, however, have been unable to replicate these findings in freshly dissociated lactotrophs. A full report of these findings is in preparation. The most parsimonious mechanism linking D_2 receptor activity to inhibition of PRL secretion would involve activation of a K^+ channel, subsequent membrane hyperpolarization to voltages where the probability of Ca^{2+} channel opening would be dramatically reduced, and consequential decrease in Ca^{2+} influx and extracellular Ca^{2+} levels leading to a decline in PRL secretion. Recently, a similar conclusion was reached by Stack & Suprenant (1991) for dopamine receptor-mediated inhibition of β-endorphin secretion from the intermediate lobe cells, where the agonist dose dependence for K^+ current activation closely correlated with that for secretion. In contrast, the reduction of Ca^{2+} currents these authors observed occurred only at much higher agonist concentrations.

Finally, a question of interest is whether a specific class of G protein is involved in the coupling of D_2 receptors to K^+ channels. While the G protein family consists of a forever growing number of proteins, experimental evidence supports the hypothesis that the endogenous G protein(s) functionally coupled to D_2 receptors in the anterior pituitary is either G_i or G_o or both (Senogles, Amklaidy, Falardeau & Caron, 1986; Ohara, Haga, Berstein, Haga & Ichiyama, 1987; Senogles *et al.* 1987, 1990). It is interesting to speculate that D_2 receptors may couple to different effector mechanisms through different specific G proteins. The G_i proteins have been shown to regulate the inhibition of adenylyl cyclase and K^+ channels (Kadata *et al.* 1984; Codina, Olate, Abramowitz, Mattera, Cook & Birnbaumer, 1988). G_o has been implicated in the regulation of Ca^{2+} and K^+ channels (Heschler, Rosenthal, Trautwein & Schultz, 1987; Harris-Warrick *et al.* 1988; VanDongen *et al.* 1988;

Ewald *et al.* 1989; Kleuss, Heschler, Ewel, Rosenthal, Schultz & Wittig, 1991). By reconstituting DA responsiveness in PTX-treated membrane patches exposed to purified G proteins, as well as blocking DA responses using specific antibodies directed against individual G proteins, future experiments will more clearly define the nature of the G proteins coupling D_2 receptors to effector K^+channels.

The authors wish to express their appreciation to Scott MacMurdo for assistance with the tissue culture and for performing the reverse haemolytic plaque assays. We also thank Dr Karen Gregerson for generation of the prolactin antiserum and instruction in the RHPA; her efforts were instrumental to the success of the project. This work was supported by National Institutes of Health grant NS18788 to G.S.O.

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